

# THE USE OF ISOTOPIC CARBON IN A STUDY OF THE METABOLISM OF ANTHRANILIC ACID IN *NEUROSPORA*\*

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The finding by Tatum, Bonner, and Beadle (1), that the *tryptophanless* *Neurospora* mutant strain 10575 accumulates anthranilic acid, which in turn can be utilized for growth of strain 40008, has provided evidence that anthranilic acid is a biochemical precursor of tryptophan in this organism. It has been further established that indole is an intermediate in this conversion (2-5).

More recent work with a number of mutants of *Neurospora* (6-8) has established that tryptophan is a biochemical precursor to niacin with kynurenine and hydroxyanthranilic acid as intermediates. The accumulated evidence has indicated the existence in the mold of the following series of reactions:

→ Anthranilic acid → indole → tryptophan → kynurenine → 3-hydroxy-anthranilic acid → nicotinic acid

In the light of this evidence the present work was undertaken to trace the carbon in the carboxyl group of anthranilic acid in order to estimate its contribution as a structural unit in the formation of niacin and tryptophan. The organism chosen for this investigation was a biochemical mutant strain of *Neurospora* designated as strain 40008. This mutant utilizes anthranilic acid, indole, or tryptophan for growth.

The mutant was grown in the presence of anthranilic acid containing  $C^{14}$  in the carboxyl group. Niacin and tryptophan were isolated from the mold mycelium and tested for radioactivity.

## EXPERIMENTAL

*Growth of Mold*—*Neurospora* mutant 40008 was grown under forced aeration for 6 days at room temperature in 10 liters of minimal medium

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(9) to which a supplement of 16.7 mg. of active anthranilic acid was added. A trap containing 40 per cent NaOH was connected to the system to absorb any carbon dioxide evolved during growth of the mold. At the end of the growth period the mold was filtered off, and the moist mycelium was continuously extracted with 300 ml. of acetone for 20 hours. The dried residual mycelium weighed 3.7 gm.

*Isolation of Niacin*—The acetone extract of the mycelium was taken to dryness, redissolved in 50 ml. of water, and filtered. It contained 700  $\gamma$  of nicotinic acid or its amide.<sup>1</sup> After removing a 4 per cent aliquot for further study, 4 gm. of anhydrous barium hydroxide were added to the main portion of the extract, and it was heated at 90° for 40 minutes, titrated to pH 6.5 with 2 N H<sub>2</sub>SO<sub>4</sub>, and filtered. The filtrate was taken to pH 4.2 with concentrated HCl and shaken with 1 gm. of norit A. This was filtered off and washed well with water.

The charcoal was eluted with two 50 ml. portions of hot 4 per cent aniline in water. The combined eluates were extracted with ether to remove the aniline and taken to dryness. The residue was taken up in 13.5 ml. of hot absolute alcohol and filtered. After readjusting the volume of the filtrate to 13.5 ml., a 1.5 ml. portion was taken for bioassay and investigation with paper chromatography. The total filtrate contained, according to bioassay, 300  $\gamma$  of nicotinic acid. This material was established as nicotinic acid by comparing its movement on an ascending paper chromatogram with that of an authentic sample of nicotinic acid. Butanol saturated with water was the solvent used for developing the chromatograms.

40 mg. of nicotinic acid were added to 12 ml. of the original 13.5 ml. of filtrate containing the isolated nicotinic acid. The nicotinic acid which precipitated from a concentrated aqueous solution was recrystallized from a 1:4 mixture of acetic acid and benzene. The isolated nicotinic acid melted at 229–232° and gave the same ultraviolet absorption spectrum as an authentic sample of the same acid.

*Isolation of Tryptophan*—10 mg. of pepsin (1:10,000 potency) were added to the 3.7 gm. of ground dry mycelium suspended in 50 ml. of 0.1 N H<sub>2</sub>SO<sub>4</sub>. The mixture was covered with toluene and incubated 21 hours at 37°. At the end of the first digestion 3 gm. of K<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O were added and the pH was adjusted to 8.4. The mixture was incubated at 40° for 72 hours in the presence of 50 mg. of trypsin (1:300 potency). The undigested suspended material was filtered off and washed with water. The combined filtrate and washings, diluted to 100 ml. with water, contained 13.2 mg. of tryptophan. The identity of this tryptophan was

<sup>1</sup> *Lactobacillus arabinosus* strain 17-5 was used to determine nicotinic acid and tryptophan.

confirmed by comparing its position on an ascending paper chromatogram with that of an authentic tryptophan sample. Water-saturated butanol was used as the solvent for the chromatogram.

To 20 ml. of the original 100 ml. of enzyme digest were added 20 mg. of stock L-tryptophan and 3 ml. of 50 per cent sulfuric acid. The mercury salt of tryptophan was precipitated by the addition of 2 gm. of  $\text{HgSO}_4$  in 10 ml. of 6 per cent sulfuric acid. The mixture was allowed to stand 48 hours, at the end of which time the yellow precipitate was filtered off, washed with 5 per cent sulfuric acid, and finally with water. The moist mercury precipitate was suspended in 8 ml. of water and made alkaline to

TABLE I  
*Distribution of Radioactivity in Mold Culture Components*

Sample No.	Component	Total counts per sec.	Activity* per component	Activity* accounted for
			per cent	per cent
1	Culture fluid after removal of mycelium	57,500	7.2	7.2
2	Carbon dioxide trapped during growth of mold	739,652	92.3	92.3
3	Acetone-extracted material from mycelium	6,500	0.8	0.8
4	Isolated nicotinic acid	0.0	0.0	
5	Mycelium after acetone extraction	19,686	2.4	
6	Water-insoluble material after enzymatic digestion of mycelium	2,689	0.3	0.3
7	Water-soluble material after enzymatic digestion of mycelium	11,860	1.5	1.5
8	Isolated tryptophan	0.0	0.0	
Total .....				102.1

\* Based on assay of original anthranilic acid, 48,000 counts per second per mg. Total activity used =  $16.7 \times 48,000 = 801,600$  counts per second.

phenolphthalein with a concentrated barium hydroxide solution. A rapid stream of hydrogen sulfide was passed into this alkaline solution to precipitate the mercury as its sulfide. This was filtered off and washed with water. Traces of barium ion were removed by the addition of a drop of dilute sulfuric acid followed by filtration. The filtrate was taken to a pH of 5.9 with sodium carbonate, concentrated to about 5 ml. under a vacuum, and continuously extracted for 12 hours with 75 ml. of butanol. After adjusting the butanol extract back to 75 ml., a 5 ml. aliquot was taken for bioassay. The total butanol solution contained 9.4 mg. of tryptophan. The bulk of the extracted tryptophan contained in 70 ml. of butanol was taken to dryness. 40 mg. of stock tryptophan were added to the residue

and the whole was taken up in 4 ml. of water. The volume was reduced to 1.5 ml., from which the tryptophan did not precipitate, even though the solubility of the solute in that volume of water at room temperature had been greatly exceeded. Small amounts of discolored material which precipitated were filtered off. The filtrate was taken to dryness and the residue crystallized twice from 60 per cent ethanol, m.p. 273–276°. The melting point of an authentic sample of tryptophan taken simultaneously was 275–278°. The ultraviolet absorption spectrum of the isolated material was identical with that of the authentic tryptophan used for isotopic dilution.

*Distribution of Radioactivity*—A general summary of the distribution of radioactivity in the various components of the mold culture is given in Table I.

#### DISCUSSION

The absence of radioactivity in the isolated niacin and tryptophan shows that, if the carbon in the side chain of anthranilic acid was incorporated as a structural unit in the formation of these two substances in *Neurospora*, it was lost during further metabolism involving these substances. The organism used for determining the tryptophan content of the mold digest does not differentiate between anthranilic acid, indole, tryptophan, and possibly peptides of tryptophan. This ambiguity was overcome by the use of paper chromatography. Not only was the isolated tryptophan identified by its position on a chromatogram, but a separation of biological activity and radioactivity was also effected by the same technique. The problem of differentiating nicotinic acid from its amide prior to dilution was also resolved by paper chromatography. Here again, a complete separation of radioactivity from biological activity was possible.

The presence of large quantities of isotopic carbon in the carbon dioxide evolved during the growth of the mold indicates that a major portion of the carboxyl group in anthranilic acid finds its way into this substance during growth of the mold.

#### SUMMARY

1. Anthranilic acid containing  $C^{14}$  in the side chain was given to a *Neurospora* mutant form which required either anthranilic acid, indole, or tryptophan as a supplement for growth. Nicotinic acid and tryptophan isolated from the mold tissue were found to contain no detectable quantity of the isotopic carbon.

2. A large part of the isotopic carbon was accounted for in the carbon dioxide evolved during the growth of the mold.

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